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(54) Title: SPACER PEPTIDES AND MEMBRANES CONTAINING SAME

(57) Abstract

Synthetic cell membranes which are bonded to a noble metal support and which comprise novel spacer peptides of formula (I) are disclosed. These membranes can incorporate membrane proteins and can be used in biosensing devices. Peptides or peptide–analogous compounds of the formula (I) are disclosed, X–L^N–A^N–B–A^C–L^C–Y in which preferred embodiments have the following meaning: a) group X contains at least one sulphur atom, e.g. group X represents a Lip group or a group like HS–$(CH_2)_2$–CO–; groups A^N and L^N both represent single bonds; group A^C represents Pro Xaa^1 Xaa^1; group L^C represents a single bond or an oligomer (number of monomers between 2 and 4) of a medium chain ω–amino acid like 6–aminocaproic acid; group Y represents Lys(Myr)–Lys(Myr), where Myr represents a myristoyl residue bound to the side chain amino group of Lys; or b) group X represents Myr–Lys(Myr), where Myr represents myristoyl residues bound to the amino groups of Lys; group L^N represents a single bond or an oligomer (number of monomers between 2 and 4) of a medium chain ω–amino acid like 6–aminocaproic acid; groups A^N represents Pro Xaa^1 Xaa^1; groups A^C and L^C both represent single bonds; group Y contains at least one sulphur atom, e.g. group Y represents Cys–amide or group like –NH–$(CH_2)_2$–SH.

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Spacer Peptides and Membranes Containing Same

The present invention relates to lipid membranes attached to a solid support via thiopeptides as linker or spacer molecules. These lipid bilayer can incorporate a plurality of membrane proteins such as ion channels, ionophores and integral membrane proteins such as ion pumps and receptors and can be used in biosensing devices, when the ion flux is modulated by agonists, antagonists, drugs, substrates and ligands. Such a system is useful as a screening test for pharmaceutically active compounds.

Polymeric spacers have been used to prepare such films, particularly polyoxiethylene spacers (US 5,401,378 or WO 92/17 788) also attached to the gold support via Au-S-groups. These spacers are, however, not designed to form secondary structures such as helices and therefore, they are less rigid and more ready to collapse compared to peptides. (Layer thicknesses are not reported) Lipid films with polyoxiethylene spacers, therefore, were shown to incorporate ion channels and ionophores such as alamethicin, gramicidin and valinomycin. Ion transport through these channels was measured by impedance spectroscopy. However, membrane spanning proteins with large domains extending in the aqueous phase such as receptors and ion pumps were incorporated only rarely and not preserving the activity of these proteins. Peptide spacers tethered to the gold support via -S-S- or -S-H groups are disclosed in WO 96/18 645 and DE 44 44 893.7. They were shown to be covalently linked in situ to a lipid (DMPE) to form peptide spacered lipid monolayers. These monolayers in contact to a lipid vesicle suspension spontaneously formed peptide supported lipid bilayers. One way to incorporate membrane proteins had been shown to be the fusion of vesicles containing the reconstituted protein, whereby their activity was being preserved. The peptide spacer had therefore proved to be compatible with the integral protein. Because of the poor solubility of the thiopeptide disclosed in WO 96/18 645 and DE

44 44 893.7 the synthesis of the thiopeptide-lipid adduct in the bulk was not possible. It had therefore been carried out in-situ on the gold support. The electrical properties of the system were also not satisfying. In order to remove these shortcomings the system has to be improved with respect to:

- the peptide sequence;
- the preparation procedures of the peptide-lipid adduct (in-situ and ex-situ);
- the experimental set-up of the electrochemical and optical measurements;
- preparation of the thiopeptide and peptide lipid monolayer
- the (*ex-situ)* preparation of the peptide lipid adduct in the bulk
- demonstration of the self assembly of this thiopeptide-lipid adduct on the gold support to form a stable monolayer
- fusion of liposomes to form the respective lipid bilayer to the thiopeptide-lipid adduct
- the incorporation of other membrane spanning proteins
- demonstrating and measuring their binding properties as well as their activity
- demonstrating the appropriate methods of measurement, e.g. impedance spectroscopy, surface plasmon fluorescence spectroscopy

Thus the invention is based on the object of finding novel compounds with improved properties, in particular those which can be used to prepare peptide layers, peptide-analogous layers, peptide-lipid layers or cell membranes or devices containing such membranes.

The present invention relates to lipid membranes attached to a solid support via thiopeptides as linker or spacer molecules. As illustrated in fig. 1. these films are formed from a thiopeptide described below which is covalently attached on one side to the gold support via Au-S- bonds and on the other to a phospholipid (DMPE) via CO-NH-bonds, thus forming a thiopeptide-lipid monolayer. In contact with a suspension of liposomes the

peptide lipid monolayer spontaneously forms the respective lipid bilayer separated from the support by an aqueous phase. The lipid bilayer incorporates a plurality of membrane proteins such as ion channels, ionophores and integral membrane proteins such as ion pumps and receptors. The ion flux through these molecules can be monitored and is characteristic for the activities of these proteins. In such a way, these supported lipid bilayers are interesting as model systems for biological membranes. They are particularly useful when membrane spanning proteins are incorporated in the lipid film. Then they can be used as biosensors or systems for bioassays when the ion flux is modulated by agonists, antagonists, drugs, substrates and ligands. The binding properties of the membrane proteins, for example towards antibodies and ligands, can be assayed simultaneously together with the ion flux. Unspecific binding is reduced due to the lipid environment. Such a system could be used as a screening test for pharmaceutically active compounds.

The invention relates to peptides or peptide-analogous compounds of the formula I

$$X - L^N - A^N - B - A^C - L^C - Y \qquad I$$

in which

X is

a diamino acid, which can be acylated once or twice by an acyl moiety with 1 to 22 C-atoms

or

a sulphur containing residue like a 1,2-Dithiolane-3-pentanoyl (lipoyl) residue or HS-alkyl-CO- or HS-alkyl-CO-NH-alkyl'-CO- or Trt-S-alkyl-CO- or Trt-S-alkyl-CO-NH-alkyl'-CO- or Cys, which can be acylated once by an acyl moiety with 2 to 18 C-atoms

or

H;

L^N is a single bond

or

1 to 5 ω-amino acid moieties connected by peptide bonds, wherein the amino acid moieties contain 3 to 10 carbon atoms;

A^N is

a single bond

or

Xaa^1 Xaa^1 Pro

B is

Xaa^2 Xaa^1 Xaa^1 Xaa^2 Xaa^2 Xaa^1 Xaa^2

A^C is

a single bond

or

Pro Xaa^1 Xaa^1;

L^C is a single bond

or

1 to 5 ω-amino acid moieties connected by peptide bonds, wherein the amino acid moieties contain 3 to 10 carbon atoms;

and

Y is

a sulphur containing residue like Cys, the carboxyl group of which optionally can be substituted by -OAlk, $-NH_2$, -NHAlk or $-NAlk_2$, or $-HN-(CH_2)_n-SH$, with n = 2-11

or

an diamino acid like Lys, Orn, Dpr, or Dbu, optionally the side chain amino group of these diamino acids can be acylated by an acyl moiety with 2 to 22 C-atoms;

or

a dipeptide containing two diamino acids like Lys, Orn, Dpr, or Dbu, optionally one or both side chain amino groups of these diamino acids can be acylated by an acyl moiety with 2 to 22 C-atoms

or

OH;

and

Alk and Alk´

are independent of each other

a straight chain or branched alkyl with 1 to 11 C-atoms,

Pro can also be 3Hyp, or 4Hyp;

Xaa^1 is a hydroxy amino carbonic acid with 3 or 4 C-atoms, e.g. Ser, Thr, allo-Threonine, homo-Serine;

Xaa^2 is a 2-alkylglycine with C_1 - C_5 alkyl (straight chain or branched), e.g. Ala, Abu, Val, Ile, or Leu;

wherein either X or Y contain at least one sulphur atom,

and wherein only of residue A^N or A^C may represent a single bond.

Two alternative structures represent preferred embodiments of the invention:

a) group X contains at least one sulphur atom, e.g. group X represents a Lip group or a group like HS-$(CH_2)_2$-CO-;

groups A^N and L^N both represent single bonds;

group A^C represents Pro Xaa^1 Xaa^1

group L^C represents a single bond or an oligomer (number of monomers between 2 and 4) of an medium chain ω-amino acid like 6-aminocaproic acid;

group Y represents Lys(Myr)-Lys(Myr), where Myr represents a myristoyl residue bound to the side chain amino group of Lys.

or

b) group X represents Myr-Lys(Myr), where Myr represent myristoyl residues bound to the amino groups of Lys;

group L^N represents a single bond or an oligomer (number of monomers between 2 and 4) of an medium chain ω-amino acid like 6-aminocaproic acid;

groups A^N represents Pro Xaa^1 Xaa^1

groups A^C and L^C both represent single bonds;
group Y contains at least one sulphur atom, e.g. group Y represents Cys-amide or group like -NH-$(CH_2)_2$-SH.

In these structures Lys can be replaced by other diamino acids as mentioned above and Myr can be replaced by other acyl moieties containing 2 to 22 C-atoms, like those mentioned later in this disclosure.

Both alternatives incorporate the same basic principle: A first terminal group containing sulphur is used to anchor the peptide to the noble metal surface. The core spacer B creates a self-organizing domain, the intermediate groups A^C and/or A^N cause the whole structure to be flexible. The other terminal group is lipophilic and inserts into the membrane structure. This structure allows to create stable membrane structures covalently bound to a noble metal surface. The alternatives mentioned above are a result of the polarity of the peptide bond.

Alternative compounds wherein the diamino acids are not acylated can be used as intermediates for synthesizing the acylated compounds mentioned in the following description.

The invention furthermore relates to processes for the production of the peptides of formula I, and to peptide layers covalently bonded via sulphur bridges of one of the terminal groups to a noble metal surface, as well as to synthetic cell membranes and complexes containing a membrane protein in said synthetic cell membrane. The invention furtermore relates to biosensing devices containing such complexes and their use for receptor binding assays and investigating the activity of pharmaceutical and crop protection agents.

The invention furthermore relates to the formation by self assembly of stable thiopeptide-lipid monolayers on top of the noble metal surface which

can be stored for a longer period of time, for example in the form of an electronic chip and which can be used at any time to form the respective lipid bilayers either by fusion with liposomes or by adding a lipid solution in a non-aqueous solution which is then diluted with an aqueous buffer. Alternatively the lipid bilayer can be stored ready to use in an aqueous environment, possibly stabilized by trehalose or glycerin present in the solution.

The preferred embodiment of such a device would be a multi-electrode chip or chip array, each electrode individually addressable, the signal of which can be monitored sequentially or by a multiplexer. The chip would contain a number of gold electrodes, each of them surrounded by a teflon sheet, preferably using a teflon mask. The purpose of the teflon sheet would be to provide a lipid reservoir and at the same time to contain the aqueous solution limiting and stabilizing the lipid bilayer.

Fig. 1 depicts schematically the forming of bilayers by reacting the lipid monolayer, which is bound via a thio peptide to the noble metal substrate (e.g. gold), with liposomes.

Fig. 2 depicts an electrochemical cell, and Fig. 3 a cell, which allows both electrochemical and optical measurements.

Fig. 4 to 6 show SPS spectra for the formation of lipid mono- and bilayers as well as the insertion of cytochrome c oxidase and the acetylcholine receptor is given in figs.4 and 5 respectively. For the binding of cytochrome c to cytochrome c oxidase, see fig.6.

Fig. 7 depicts schematically a measuring device for surface plasmon fluorescence spectroscopy (SPFS); fig. 8, 9, and 11 show the results of such measurements.

Fig. 10 depicts schematically the use of primary and secondary antibodies. Details are given below and in the examples.

Lipids and proteins are major components of biological membranes. Lipid bilayers are regarded as a model of cell membranes. Peptides or proteins can be incorporated into such lipid bilayers so that they extend through them by insertion perpendicular to the surface (J.C. Huschilt et al. BBA 979, 139-141 (1989)). The conformation of the protein-bilayer assembly is partly determined by the sequence of the peptide, for example the incorporation of membrane proteins depends on the surface charge of the peptide. Peptides useful as spacers in membranes are disclosed along with additional technical background in WO 96/18 645 and DE 44 44 893.7. The lipid component of the membranes as disclosed in WO 96/18 645 and DE 44 44 893.7 is typically bound via an ester linkage of the C-terminal amino acid. In addition to this type of structure the lipid component of the present invention can be integrated into the amino acid sequence by using

A further preferred embodiment would be a multi-electrode chip ready to form the lipid bilayer-protein assembly by fusion with liposomes or alternatively with a preformed lipid bilayer into which a membrane protein can be inserted by adding it in the solubilized form which is then diluted below the critical micelle concentration (cmc).

A further preferred embodiment would be such a bilayer-protein assembly with several interacting proteins including ion channels.

The embodiments described above can be used as biosensor devices since they respond in a reversible fashion to substrates of the membrane proteins incorporated. Alternatively, they can be used as a screening test for pharmaceutically active compounds. Membrane proteins according to the present invention and different from conventional screening tests on a microtiter plate, are placed in an environment matching very much natural

conditions. They are thus kept in the active state. Binding effects can be studied much more specifically and moreover in real time. The effect of agonists and antagonists on electrical and/or optical properties can be investigated simultaneously.

In this disclosure reference is made to gold as electrically conducting material for electrodes. However it is generally known that other noble metals like platinum, silver, or palladium or semi-noble metals like copper, as well as alloys containing such noble or semi-noble metals can be used for the same purpose. Therefore mentioning gold as material for electrodes is to be understood as example, not as an limitation. Similarly, the expression noble metals used in this disclosure is to be understood to comprise the nobel metals, as well as semi-nobel metals and alloys containing nobel metals and/or semi-nobel metals.

It has been found that mercaptoalkylcarboxy-peptides of the formula I can be bound to gold surfaces and that they organize themselves to dense layers, with improved characteristics, particularly after using the improved preparation procedure described below. Lipids (for example dimyristoyl-phosphatidyloxyethylamine, DMPE) are in-situ covalently coupled to the peptidyl-gold phase. Lipids or lipophilic residues can be covalently coupled ex-situ before the peptide is bound to the gold surface. This allows a simplified procedure for preparing the tethered lipid membranes.

The procedure described above results in producing monomolecular lipid layers which are covalently bonded via the peptide spacer group to the gold surface. Alternatively, lipid layers can also be applied to the peptide layer by the Langmuir-Blodgett technique. The method is described, for example, by G. Puu, I. Gustavson, P.-A. Ohlsson, G. Olofson and A. Sellstrøm in Progress in Membrane Biotechnologie page 279 et seq. (1991), Birkhäuser Verlag, Basel (Eds. Fernandez/Chapman/Packer).

The peptide spacer serves to form a hydrophilic layer between the hydrophobic lipid layer and the gold electrode. The additional flexible portion improves the stability of the lipid layer. The lipid monolayer formed in this way can be provided with a second lipid layer, for example with the aid of the Langmuir-Blodgett technique, by fusion of liposomes or diluting a lipid solution in a non-aqeous solvent (painted membranes), to result in defined lipid bilayers which represent a model of a biological membrane to the extent that they are adjacent to an aqueous phase on both sides. The aqueous phase adjacent to the electrode is represented by the peptide layer. It is shown to have a layer thickness corresponding to molecular dimensions of the helical conformation (N. Bunjes et al., Langmuir (1997)). The requirement of having a relatively rigid spacer which at the same time is compatible with membrane proteins is thus fulfilled. (The extension of the peptide spacer should remain in the limits of 1 to 10 nm) The aqueous layer also represents a ionic reservoir since ion transport actuated by the membrane proteins could be demonstrated and measured as an electric current which does not seem to be limited by the extent of the aqueous phase. Accordingly, lipid bilayers thus formed are shown to insert membrane proteins such as HATPase, NaKATPase, cytochrome c oxidase, and acetylcholine receptor and thus permit their electrical, structural and binding properties to be investigated.

These lipid-peptide-gold constructs are accordingly capable of forming bilayers and inserting proteins. The formation and the arrangement (order state) of the layers can be measured by cyclovoltametry, impedance spectroscopy and surface plasmon resonance spectroscopy (SPS). The binding process of antibodies, ligands, agonists, antagonists, substrates, drugs etc. to the incorporated membrane proteins can be measured in real time by SPS and more effectively by surface plasmon fluorescence spectroscopy (SPFS), and fluorescence spectroscopy. Binding constants and kinetic data can be obtained from these measurements by known procedures. Simultaneously, electrical properties of the ion flux through the proteins such as ion channels, receptors and ion pumps can be monitored by impedance spectroscopy (IS). Direct electron exchange between protein and electrode can be measured by other electrochemical techniques.

Basically SPR is known and used for biosensors (e.g. EP 0 442 922). Plasmon surface polaritons (surface plasmons for short) are excited along the metal-dielectric interface. Their field amplitudes decay exponentially, both along the interface and into the dielectricum, with the maximum intensity being at the interface.

For the present experiments a Kretschmann set-up is used. For this configuration the refractive index of the prism n_p and the angle of incidence define the x-component of the momentum of the exciting photon, where in the SPS experiment the angle of incidence is varied and the reflected intensity is detected by a photodiode. A thin dielectric coating of the metal film, e.g., a supported membrane shifts the angle of resonant coupling to surface plasmon modes to higher angles. This angular shift depends on the thickness d of the layer and its optical properties relative to the surrounding medium, i.e., on the difference between their respective indices of refraction. Examples of SPS spectra for the formation of lipid mono- and bilayers as well as the insertion of cytochrome c oxidase and the acetylcholine receptor is given in figs.4 and 5 respectively. For the binding of cytochrome c to cytochrome c oxidase, see fig.6.

Surface plasmon fluorescence is explained briefly: Plasmon surface polaritons or surface plasmons are transverse electro-magnetic waves that propagate along a metal-dielectric interface, their field amplitudes decaying exponentially perpendicular to the interface thus allowing to introduce a surface sensitive probing technique. The metal acts as an oscillator that can be driven by an electromagnetic wave impinging upon that interface (Kretschmann configuration Fig. 7). Therefore onre is dealing with the resonant excitation of a coupled state between plasma oscillations and the photons, that is, "plasmon surface polaritons". This resonance phenomenon can be clearly seen in the attenuated total reflection (Fig. 8): below some value of θ the reflectivity is very high because the metal film acts as a mirror providing little transmission. Above θ for total internal reflection, a relatively narrow dip in the reflectivity curve at θ indicates the resonant excitation of such PSP waves at the metal-dielectric interface.

Surface plasmons are well defined modes which obey the known dispersion relation. Therefore each photon of a given energy allows the excitation of exactly one PSP mode. A thin dielectric coating causes a shift of the dispersion relation to higher momentum shifting the reflectifity curve to higher angle. The simulation of this shift using a Fresnel formalism on multilayered dielectric structures determine the thickness of the dielectric film.

In addition to the resonance dip in the attenuated total reflection there is a corresponding resonant electro magnetic field enhancement located at the metal-dielectric interface. As soon as the resonance condition of the surface plasmons is established, a resonant amplification of the electro-magnetic field takes place. The intensity of this surface located field can oversize the incident intensity by a factor of 10 (gold) to 100 (silver). This intensity enhancement can be employed to increase the fluorescence emission of surface bound dye molecules excited by this amplified electro-

magnetic field. In order to avoid a significant loss of fluorescence due to Foerster transfer a spacer between the dye molecule and the metal is required providing a spatial separation of at least 5-10 nm.

The experimental detection equipment shown in Fig 7 allows to monitor reflectivity and fluorescence simultaneously. A HeNe Laser in the Kretschmann configuration is employed to excite the surface plasmons at the metal-dielectric interface. The selected laser provides the matching condition for the excitation wavelength of the selected dye molecule (CY5) yielding to the surface plasmon enhanced fluorescence emission. The temperature controlled sample is mounted on a rotary table in a theta/2 theta configuration. The reflectivity is detected by a photo diode and monitored by a lock in amplifier. The emitted fluorescence blocked by an interference filter is measured by a photo multiplier in photon counting mode mounted at the back side of the sample. A conter is employed to digitize the photo multiplier signal output. The experimental setup is driven by a PC allowing the real time simultaneous detection of reflectivity and fluorescence.

Examples of simultaneously recorded SPS and SPFS spectra of primary and secondary antibody binding to cytochrome c oxidase and acetylcholine receptor, see fig. 8, 9 and 11. The secondary antibody is fluorescence labeled with CY5.

Basic constructions for cells suitable for measuring the electrical or optical parameters mentioned above are known in the art; an example of such a measuring cell for impedance spectroscopy (IS) is described by Z. Salamon, G. Tollin, Photochem. Photobiol, **58**, 1993, p. 730-736.

An similar version of an electrochemical cell is drawn in figure 2. It comprises a Plexiglas body (1), the sample compartment (2) is lined with a teflon spacer (3) additionally provided with a sealing lip limiting the area of the gold support (4), which is used as working electrode; the gold support carries the membrane into which optionally a membrane protein can be integrated; a removable thermostated chamber beneath the support (not shown), a platinum counter electrode (5) and Ag/AgCl, saturated KCl reference electrode (6). The cell is equipped with a fiber optic bundle (7), and with a inlet (8) and outlet (9) in order to fill and empty the cell. The teflon spacer is removed and impregnated with 10 µl of a 0.5% solution of egg phosphatidyl choline in hexane prior to each measurement. Then the gold support is attached to the spacer as well as to the gold contact via the thermostated chamber. Typically the volume of the assembled cell is 800 µl.

A further improved cell which allows to measure SPS/SPFS/IS simultaneously is depicted in fig. 3: The body of the cell (11) is made from teflon. A sealing lip (12) limits the area of the gold support (13), which is used as working electrode; the gold support carries the membrane into which optionally a membrane protein can be integrated. Typically the diameter of the sealing lip is 4 mm. The inner part of the chamber (14) is formed as a conus from 4-20 mm diameter. A removable glass slide (15) covers the backside of the chamber. A thermostated chamber can be added (not shown). A platinum counter electrode and Ag/AgCl, saturated KCl reference electrode are inserted in the chamber via two tubes (16) and (17).

A schematic view of a measuring device for measuring SPFS is depicted in Fig. 7. Compared to SPS-measurements the SPFS-measurement improves the sensitivity. Sensitivity can be further improved by using a second antibody labeled with a fluorochrome. A schematic view of a membrane protein labeled with a first antibody, to which in turn a second antibody is bound is depicted in Fig. 9. The second antibody is labeled with a fluorochrome.

The compounds of the formula I can be employed as building blocks for synthetic peptide layers or biological membranes, in particular cell membranes. They can be bound to a nobel metal support. Into such tethered membranes membrane proteins can be integrated by processes known in the art. The resulting complex of tethered membrane and membrane protein can be used in a biosensing device for measuring analytes (biosensor) and for measuring interactions of the membrane proteins with reactants in solution such as a bioassay for a drug screening test. Examples for membrane proteins, which can be integrated into membranes are: Cytochrome c oxidase (COX), ATPases from different sources (e.g. H^+-ATPase from plant chloroplasts or Na/K-ATPase from shark), acetylcholine receptor from *Torpedo californica*,) and integrins like e.g. Integrin $\alpha_v\beta_{III}$. Other membrane proteins are known in the art. These complexes can be used in devices for measuring specific binding assays for antibodies and for other electrochemical and optical measurements in order to quantify biological interactions.

The abbreviations of amino acid residues mentioned hereinbefore and hereinafter represent the residues of the following amino acids:

Abu 2-aminobutyric acid

Acp 6-aminocaproic acid

Ala	alanine
Arg	arginine
Asp	aspartic acid
Cys	cysteine
Dbu	2,4-diamino butyric acic
Dpr	2,3-diamino propionic acid
Gly	glycine
Hcy	homo cysteine
Hse	homo serine
3Hyp	3-Hydroxyproline
4Hyp	4-Hydroxyproline
Leu	leucine
Ile	isoleucine
Lys	lysine
Orn	ornithine
Pro	proline
Ser	serine
Val	valine
Thr	threonine
Xaa^1	hydroxy amino carbonic acid with 3 or 4 C-atoms, e.g. Ser, Thr, allo-Threonine, homo-Serine.
Xaa^2	2-alkylglycine, wherein the alkyl group contains 1 to 5 C-atoms and can be straight or branched, e.g. Ala, Abu, Val, Ile, or Leu

Further meanings hereinafter are:

alkyl	C_1 to C_{11} alkyl or alkylene (straight chain or branched)
BOC	tert-butoxycarbonyl
CBZ	benzyloxycarbonyl
DCCI	dicyclohexylcarbodiimide
DIC	diisopropylcarbodiimide

DMF	dimethylformamide
DMPE	dimyristoylphosphatidylethanolamine
EDCI	N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride
Et	ethyl
Fmoc	9-fluorenylmethoxycarbonyl
HOBt	1-hydroxybenzotriazole
Lau	lauroyl
Lip	1,2-Dithiolane-3-pentanoyl (lipoyl)
Me	methyl
MBHA	4-methylbenzhydrylamine
Mtr	4-methoxy-2,3,6-trimethylphenylsulfonyl
Myr	myristoyl
OBut	tert-butyl ester
OMe	methyl ester
OEt	ethyl ester
POA	phenoxyacetyl
TFA	trifluoroacetic acid
Trt	trityl (triphenylmethyl).

The ω-amino acid moieties which form the portions L^N respectively L^C contain 3 to 10 carbon atoms; examples for suitable ω-amino acids are ß-alanine, 4-aminobutyric acid, and 6-aminocaproic acid.

Examples of acyl residues with with 2 to 22 C-Atomes are: acetyl, propanoyl, butyryl, caproyl, preferred embodiments of these acyl residues are myristoyl, lauroyl, palmitoyl, and stearoyl, as well as their unsaturated counterparts.

Where the abovementioned amino acids or residues thereof are able to occur in several enantiomeric forms, all these forms and also their mixtures (for example the DL forms) are included hereinbefore and hereinafter, for example as constituent of compounds of the formula I. It is furthermore

possible for the amino acids or the amino acid residues to be derivatized in a form known per se.

The invention furthermore relates to a process for preparing a compound of the formula I or one of its salts, whereby it is liberated from one of its functional derivatives by treatment with a solvolyzing or hydrogenolyzing agent. These functional derivatives can be obtained in analogous manner as disclosed in WO 96/18 645 and DE 44 44 893.7.

If X is containing sulphur it is preferably Trt-S-alkyl-CO, HS-alkyl-CO-, Trt-S-alkyl-CO-NH-alkyl'-CO-, HS-alkyl-CO-NH-alkyl'-CO-, 1,2-Dithiolane-3-pentanoyl- (lipoyl-), N-acetyl cysteine, or N-acetyl homocysteine. In these formulae, alkyl and alkyl' are, independently of one another, preferably $-CH_2-$, $-(CH_2)_2-$, $-(CH_2)_3-$, $-(CH_2)_4-$, $-(CH_2)_5-$, $-(CH_2)_9-$, $-(CH_2)_{10}-$ or $-(CH_2)_{11}-$. If X is related to a diacylated diamino acid it is preferably N,N´-dimyristoyl lysine, N,N´-dilauroyl lysine, or N,N´-dipalmitoyl lysine, N,N´-dimyristoyl ornithine, N,N´-dilauroyl ornithine , or N,N´-dipalmitoyl ornithine, or N,N´-dimyristoyl, N,N´-dilauroyl, or N,N´-dipalmitoyl derivatives of other diamino acids listed above. If Y is related to an acylated diamino acid it is preferably N´-myristoyl lysine, N´-lauroyl lysine, or N´-palmitoyl lysine, N´-myristoyl ornithine, N´-lauroyl ornithine , or N´-palmitoyl ornithine, or N´-myristoyl, N´-lauroyl, or N´-palmitoyl derivatives of other diamino acids listed above.

Particularly suitable compounds of the formula I are:

(a) $HS-(CH_2)_2$-CO-Ala-Ser-Ser-Ala-Ala-Ser-Ala-Pro-Ser-Ser-OH;

(b) $HS-(CH_2)_2$-CO-Ala-Ser-Ser-Ala-Ala-Ser-Ala-Lys-Lys-OH;

(c) Ser-Ser-Pro-Ala-Ser-Ser-Ala-Ala-Ser-Ala-Cys-amide;

(d) MyrLys(Myr)-Ser-Ser-Pro-Ala-Ser-Ser-Ala-Ala-Ser-Ala-Cys-amide;

(e) $HS-(CH_2)_2$-CO-Ala-Ser-Ser-Ala-Ala-Ser-Ala-Lys(Myr)-Lys(Myr)-OH;

(f) Ala-Ser-Ser-Ala-Ala-Ser-Ala-Pro-Ser-Ser-Cys-NH_2

(g) Ala-Ser-Ser-Ala-Ala-Ser-Ala-Pro-Ser-Ser-NH-$(CH_2)_2$-SH

(h) Myr-Lys(Myr)-Ala-Ser-Ser-Ala-Ala-Ser-Ala-Pro-Ser-Ser-Cys-NH_2

(i) Myr-Lys(Myr)-Ala-Ser-Ser-Ala-Ala-Ser-Ala-Pro-Ser-Ser-NH-$(CH_2)_2$-SH

(k) Ser-Ser-Pro-Ala-Ser-Ser-Ala-Ala-Ser-Ala-Cys-amide

(l) Ser-Ser-Pro-Ala-Ser-Ser-Ala-Ala-Ser-Ala-NH-$(CH_2)_2$-SH

(m) Myr-Lys(Myr)-Ser-Ser-Pro-Ala-Ser-Ser-Ala-Ala-Ser-Ala-Cys-amide

(n) Myr-Lys(Myr)-Ser-Ser-Pro-Ala-Ser-Ser-Ala-Ala-Ser-Ala-NH-$(CH_2)_2$-SH

(o) Lip-Ala-Ser-Ser-Ala-Ala-Ser-Ala-Pro-Ser-Ser

(p) Lip-Ala-Ser-Ser-Ala-Ala-Ser-Ala-Pro-Ser-Ser-Lys-Lys

(q) Lip-Ala-Ser-Ser-Ala-Ala-Ser-Ala-Pro-Ser-Ser-Lys(Myr)-Lys(Myr)

(r) HS-$(CH_2)_2$-CO-Ala-Ser-Ser-Ala-Ala-Ser-Ala-Pro-Ser-Ser-Acp-Acp-Acp-Lys-Lys

(s) HS-$(CH_2)_2$-CO-Ala-Ser-Ser-Ala-Ala-Ser-Ala-Pro-Ser-Ser-Acp-Acp-Acp-Lys(Myr)-Lys(Myr)

(t) HS-$(CH_2)_2$-CO-Ala-Ser-Ser-Ala-Ala-Ser-Ala-Pro-Ser-Ser-Lys(Myr)-Lys(Myr)

and as far as applicable, the salts thereof.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The preferred specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the disclosure in any way whatsoever.

The entire disclosures of all applications, patents, and publications cited above and below, and of corresponding European application EP 97118326.4 filed October 22, 1997, are hereby incorporated by reference.

Examples

Reagents (reagent grade) were purchased from Merck KGaA unless stated otherwise. Peptides are purified by gel filtration and/or HPLC as needed.

Gold substrates to be used for Surface Plasmon Resonance Spectroscopy (SPS) are deposited directly on high refractive index glass LaSFN9 (Berliner Glas) substrates (2.5*4*0.2 cm) by electrothermal evaporation to a thickness of 50 nm.

Unless otherwise indicated all SPS investigations were done at T = 30 °C with the lipid layers being in a fluid state. As measuring cell, a 4x4x0.5 cm Quartzglass with an hemisphere gap with two pipes and a polished surface (to prevent leaking) was used. The cell was cleaned by sonification in Hellmanex, ethanol and MQ water.

Gold substrates to be used for electrochemical and FTIR measurements are prepared as follows: ELKA microscope glass slides (76 * 22 mm) are cleaned. Subsequently gold is deposited to a layer thickness of 500 nm over a sublayer of 30 nm chromium in a spoon-like pattern to form 8 electrodes per slide. Chromium and gold are deposited by electrothermal evaporation using a Leybold-Heraeus L 650 vapour deposition apparatus at 300 °C and a pressure of 10^{-5} bis 10^{-6} mbar. Gold substrates are coated with thiopeptide directly after the evaporation process to prevent soiling.

Example 1: Preparation of the thiopeptide HS-$(CH_2)_2$-CO-Ala-Ser-Ser-Ala-Ala-Ser-Ala-Pro Ser-Ser-OH

HS-$(CH_2)_2$-CO-Ala-Ser-Ser-Ala-Ala-Ser-Ala-Pro Ser-Ser-OH is obtained by solid phase peptide synthesis in a continuous flow synthesizer using Fmoc (9-fluorenyl-methoxycarbonyl) strategy with acid labile side chain protection on an acid labile Wang-resin. Washes are done with DMA

(dimethylaminoacetamide), cleavage of the Fmoc-protection group is achieved with 20% piperidin in DMF. Triple couplings of 1.3 equivalents each with DIC/HOBt (diisopropylcarbodiimide/N-hydroxobenzotriazole) for 20 min are followed by capping with Ac_2O/pyridine/DMF 2/3/15 (vol.) for 10 min. The synthesis is monitored continuously by UV-spectroscopy at 310 nm. Loading of the starting amino acid and peptide bound to the resin is quantified by Fmoc-determination. After removal of the Fmoc-groups the peptide on the resin is modified at the N-terminus with tritylmercapto-propionic acid using the normal coupling procedure. Tritylmercapto-propionic acid is obtained by tritylation of mercatopropionic acid in dichloromethane with excess of triphenylmethanol and TFA catalysis at room temperature. The peptide is removed from the resin and its side chain protection groups are cleaved by treatment with TFA/CH_2Cl_2/anisol 60/40/1 (20 ml/g resin) for 2-4 h at room temperature. To remove trityl functions TFA/CH_2Cl_2/thiophenol 70/20/10 is used instead. The filtrate is concentrated and the peptide precipitated with ether. Purification is achieved by gel filtration in isopropanol/water 80/20 with 0.05% TFA on Sephadex G10 and HPLC on Lichrosorb RP 8 in 0.3% TFA with a gradient of 0-80% 2-propanol. Purity of the peptides is determined by HPLC, to be usually better than 95% and identity is found as expected by FAB-MS (fast atom bombardment mass spectroscopy).

Example 2: Preparation of the thiopeptide Myr-Lys(Myr)-Ser-Ser-Pro-Ala-Ser-Ser-Ala-Ala-Ser-Ala-Cys-NH_2

The peptide sequence Ser-Ser-Pro-Ala-Ser-Ser-Ala-Ala-Ser-Ala-Cys-NH_2 is assembled by solid phase peptide synthesis in a continuous flow synthesizer using Fmoc (9-fluorenyl-methoxycarbonyl) strategy with acid labile side chain protection on an acid labile amino xanthenyl resin (obtained from Novabiochem). Ser(But) and Cys(Trt) derivatives are used. Washes are done with DMA (dimethylaminoacetamide), cleavage of the Fmoc-protection group is achieved with 20% piperidin in DMF. Triple

couplings of 1.3 equivalents each with DIC/HOBt (diisopropylcarbodiimide/N-hydroxobenzotriazole) for 20 min are followed by capping with Ac_2O/pyridine/DMF 2/3/15 (vol.) for 10 min. The synthesis is monitored continuously by UV-spectroscopy at 310 nm. Loading of the starting amino acid and peptide bound to the resin is quantified by Fmoc-determination. After removal of the Fmoc-groups the peptide on the resin is modified at the N-terminus with Fmoc-Lys(Fmoc)-OH using the normal coupling procedure. The Fmoc groups are removed again and the capping procedure is used with myristoic acid chloride instead of acetic anhydride to derivatise both amino functions of the N-terminal lysine. The peptide is removed from the resin and its side chain protection groups are cleaved by treatment with TFA/CH_2Cl_2/anisol 60/40/1 (20 ml/g resin) for 2-4 h at room temperature. To remove trityl functions TFA/CH_2Cl_2/thiophenol 70/20/10 is used. The filtrate is concentrated and the peptide Myr-Lys(Myr)-Ser-Ser-Pro-Ala-Ser-Ser-Ala-Ala-Ser-Ala-Cys-NH_2 precipitated with ether. Purification is achieved by gel filtration in isopropanol/water 80/20 with 0.05% TFA on Sephadex G10. Purity of the peptides is determined by HPLC, to be usually better than 95%. Identity is found as expected by FAB-MS (fast atom bombardment mass spectroscopy).

Example 3: Preparation of the thiopeptide Myr-Orn(Myr)-Ala-Ser-Ser-Ala-Ala-Ser-Ala-Pro-Ser-Ser-NH-Et-SH

The peptide sequence Ala-Ser-Ser-Ala-Ala-Ser-Ala-Pro-Ser-Ser-NH-Et-SH is assembled by solid phase peptide synthesis in a continuous flow synthesizer using Fmoc (9-fluorenyl-methoxycarbonyl) strategy with acid labile side chain protection on cysteamine 2-chloro trityl resin (obtained from Novabiochem). Ser(But) derivatives are used. Washes are done with DMA (dimethylaminoacetamide), cleavage of the Fmoc-protection group is achieved with 20% piperidin in DMF. Triple couplings of 1.3 equivalents each with DIC/HOBt (diisopropylcarbodiimide/N-hydroxobenzotriazole) for 20 min are followed by capping with Ac_2O/pyridine/DMF 2/3/15 (vol.) for 10

min. The synthesis is monitored continuously by UV-spectroscopy at 310 nm. Loading of the starting amino acid and peptide bound to the resin is quantified by Fmoc-determination. After removal of the Fmoc-groups the peptide on the resin is modified at the N-terminus with Fmoc-Orn(Fmoc)-OH using the normal coupling procedure. The Fmoc groups are removed again and the capping procedure is used with myristoic acid chloride instead of acetic anhydride to derivatise both amino functions of the N-terminal ornithine. The peptide is removed from the resin and its side chain protection groups are cleaved by treatment with TFA/CH_2Cl_2/thiophenol. The filtrate is concentrated and the peptide Myr-Orn(Myr)-Ser-Ser-Pro-Ala-Ser-Ser-Ala-Ala-Ser-Ala-NH-Et-SH precipitated with ether. Purification is achieved by gel filtration in isopropanol/water 80/20 with 0.05% TFA on Sephadex G10. Purity of the peptides is determined by HPLC, to be usually better than 95%. Identity is found as expected by FAB-MS (fast atom bombardment mass spectroscopy).

Example 4: in-situ Preparation of a thiopeptide-lipid monolayer

A gold substrate is incubated for 5 hours in a solution of the thiopeptide (0.4 mg/ml) obtained in Example 1 dissolved in DMF containing 8 mg/ml LiCl. Then the gold substrate is rinsed with DMF, water, and ethanol. The slides are dryed under a stream of nitrogen and placed in a solution of 1 mg/ml DMPE (SIGMA) dissolved in a mixture of $CHCl_3$ and 2-propanol (4:1; v:v) supplemented with 0.25 mg/ml HOBt. The terminal carboxy groups of the peptide are activated by reaction with DIC (20 µl/ml; SIGMA) and coupled by additional incubation with N-ethyl diisopropylamine (10 µl/ml). Activation and coupling are once more repeated using fresh reagents. The substrate is then rinsed with $CHCl_3$, water and dried in a stream of nitrogen.

The resulting slides containing lipid monolayers were cut into single electrodes.

Example 5: Preparation of the thiopeptide bilayer from the thiopeptide lipid adduct

The substrates are incubated for 12 h in a solution (0.5 mg/ml) of the thiopeptide lipid (MyrLys(Myr)-Ser-Ser-Pro-Ala-Ser-Ala-Ser-Ala-Cys-amide) in TFA, obtained according to Example 2. Subsequently rinsed with TFA, water, and ethanol, and are then dried under a stream of nitrogen.

The TFA coating solution of the thiopeptide may also contain thioethanol or the -S-S- dimer of thioacetic acid and/or free phospholipids in order to provide additional hydrophilic groups to accomodate free lipids in the array of covalently bound thiolipids. This is an additional or alternative means with regard to impregnating the teflon spacer with free phospholipid in order to improve the arrangement and fluidity of the thiopeptide supported bilayer.

Liposomes are prepared by dialysis from phophatidylcholine from egg yolk (Lipoid E PC; 8 mg/ml), and cholesterol (30%) where indicated. The average diameter of the vesicles is 150 nm. They are expected to be large unilamellar vesicles (LUVs), i.e. equilibrated with respect to osmotic pressure.

Substrates prepared as described above are incubated at 30° C overnight in suspensions of the liposomes prepared as described above (0.8 mgl/ml) with the lipid monolayers. Subsequently substrates are rinsed and placed in fresh buffer solution (100 mM K-HEPES, pH7,4, 40 mM KCl, sterilized by filtration).

Example 6: Incorporation and activity measurements of the Cytochrome c oxidase from horse heart

Cytochrome c oxidase inserts spontaneously into a preformed thiopeptide lipid bilayer, when the protein in the solubilized form (attached to cholate as described by Kadenbach et al. Methods Enzymol. (1986) 126, 32) is diluted below the critical micelle concentration on a lipid bilayer prepared of thiopeptide Myr-Lys(Myr)-Ser-Ser-Pro-Ala-Ser-Ser-Ala-Ala-Ser-Ala-Cys-NH_2 . This process was followed by SPS as shown fig. 4. The thickness of the bilayer increased from about 6nm to 12nm. The activity was monitored by IS. The resistance of the bilayer with cytochrome c oxidase incorporated dropped from 200kOhm to 17kOhm when cytochrome c in a concentration of 8µmol/L was added as a substrate. Removal of cytochrome c (by rinsing) or addition of cyanide as inhibitor restored the resistance back to the original value, see fig. 12. Results of single IS measurements are given in Table 1.

Table1:

Electrical parameters obtained from measured Re(Z)/ω plots

	R/MΩ cm	τ/sec	α	C/μF cm^{-1}
Lipid monolayer	2.54	67	0.072	26.4
Lipid bilayer	0.883	13.3	0.23	15.1
Lipid bilayer with COX	0.747	8.83	0.132	11.8
Lipid bilayer with COX +50 mV, - cytochrome c	0.23	2.5	0.127	10.7
dito -100 mV	0.19	2.9	0.207	15.2
dito -150 mV	0.13	1.68	0.171	13.3
dito -200 mV	0.068	0.74	0.113	10.9
lipid bilayer with COX + 50 mV + cytochrome c	0.033	0.49	0.286	14.7
dito -100 mV	0.035	0.50	0.269	14.2
dito -150 mV	0.035	0.52	0.257	15.0
dito -200 mV	0.032	0.38	0.232	11.9
Lipid bilayer with COX -200 mV - cytochrome c	0.068	0.74	0.113	10.9
dito + 8 μmol/l cytochrome c	0.064	0.67	0.113	10.5
dito + 28 μmol/l cytochrome c	0.043	0.53	0.166	12.5
dito + 48 μmol/l cytochrome c	0.036	0.44	0.184	12.6
dito + 128 μmol/l cytochrome c	0.032	0.39	0.225	12.0
dito + 208 μmol/l cytochrome c	0.032	0.38	0.231	11.8
Lipid bilayer with COX -200 mV	0.078	0.79	0.045	10.1
dito + 4 μmol/l cytochrome c	0.058	0.60	0.073	10.4
dito + 8 μmol/l cytochrome c	0.026	0.29	0.197	10.7
dito + 12 μmol/l cytochrome c	0.026	0.25	0.141	9.6
dito - cytchrome c	0.079	0.92	0.080	11.7
Lipid bilayer with COX -200 mV	0.195	1.64	0.084	8.4
dito + 12 μmol/l cytochrome c	0.044	0.35	0.105	7.9
+ 100 μmol/l CN^-	0.104	0.83	0.080	8.0
+ 200 μmol/l CN^-	0.124	1.05	0.091	8.5
+ 600 μmol/l CN^-	0.15	1.44	0.120	9.7

The successful incorporation and functional orientation from the incorporated protein was proofed via SPFS. A primary monoclonal antibody against subgroup IV of the cytochrome c oxidase was added to the system, and detected with an secondary polyclonal antibody with a fluorescence label. The resulting fluorescence signal was measured in the SPFS configuration, see fig. 7.

Example 7: Incorporation and activity measurements of the acetylcholine receptor (AChR) from *Torpedo Californica*

The AChR was incorporated into the lipid film (prepared from thiopeptide lipid (MyrLys(Myr)-Ser-Ser-Pro-Ala-Ser-Ala-Ser-Ala-Cys-amide) in TFA, obtained according to Example 2) by fusion of liposomes containing the incorporated receptor, fig.5.

Preparation of the vesicles: 180 mg Av20 + 20mg Cholesterol were diluted in chloroform. The chloroform is removed in an rotary evaporator with subsequent lyophyllisation for 12h. 10ml buffer (500mM NaCl, 10mM HEPES pH 7,4, 2mM $CaCl_2$, 0,02% NaN_3) are added. After sonification for 1min and 5 frozen/thaw cycles, the vesicles were extruded with an 400nm polycarbonate filter (11 times) frozen with liquid nitrogen and stored under -70°C.

The vesicles were added to the thiopeptide lipid layer, the fusion process was followed by SPS, see fig.5. The thickness of the monolayer increased from about 4nm to 9nm. The activity was monitored by IS as shown in fig.13. The resistance dropped from 300kOhm to 10kOhm when acetylcholine in a concentration of 1µmol/L was added as a substrate. The successful incorporation and functional orientation from the incorporated protein was proofed via SPFS. A primary monoclonal antibody against the receptor was added to the system, and detected with an secondary polyclonal antibody with a fluorescence label. The resulting fluorescence signal was measured in the SPFS configuration, see fig.7.

Example 8: Incorporation and activity measurements of the Na^+K^+ATPase from shark

The ATPase inserts spontaneously into a preformed thiopeptide lipid bilayer, prepared from the thiopeptide lipid (MyrLys(Myr)-Ser-Ser-Pro-Ala-

Ser-Ala-Ser-Ala-Cys-amide in TFA, obtained according to Example 2. When the protein in the solubilized form is diluted below the critical micelle concentration. (solubilized in E_8C_{18}) The activity was monitored by IS, the resistance dropped from 200 kOhm to 20 kOhm, fig.14.

Table 2: Thickness, measured by SPS of thiopeptide-supported lipid mono- and bilayers, compared to lateral dimensions of the molecules according to molecular modelling calculations

Layer	Thickness/nm measured by SPS*			
	Peptide A	Peptide B	Peptide C	Peptide D
Thiopeptide-Monolayer	12±1	12	10	n.d.
Thiopeptide - Lipid Monolayer	27±4	39	n.d.	33
Thiopeptide - Lipid Bilayer	51±3		70±10	60
Cytochrome C Oxidase, spontaneous incorporation	171±5			180
Acetylcholine Receptor, vesicle spreading	115±10	116±10		

* optical parameters: Au: d = 493 nm, ε' = -12.657, ε'' = 1.461, peptide: n = 1.41, lipid: n = 1.5

Peptide A: SH-$(CH_2)_2$-CO-Ala-Ser-Ser-Ala-Ala-Ser-Ala

Peptide B: SH-$(CH_2)_2$CO-Ala-Ser-Ser-Ala-Ala-Ser-Ala-Lys-Lys

Peptide C: SH-$(CH_2)_2$CO-Ala-Ser-Ser-Ala-Ala-Ser-Ala-Pro-Ser-Ser

Peptide D: Myr-Lys-(Myr)-Ser-Ser-Pro-Ala-Ser-Ser-Ala-Ala-Ser-Ala-Cys-amide

Claims

1. Peptide or peptide-analogous compound of formula I,

$$X - L^N - A^N - B - A^C - L^C - Y \qquad I$$

in which

X is

a diamino acid, which can be acylated once or twice by an acyl moiety with 1 to 22 C-atoms

or

a sulphur containing residue like a 1,2-Dithiolane-3-pentanoyl (lipoyl) residue or HS-alkyl-CO- or HS-alkyl-CO-NH-alkyl'-CO- or Trt-S-alkyl-CO- or Trt-S-alkyl-CO-NH-alkyl'-CO- or Cys, which can be acylated once by an acyl moiety with 2 to 22 C-atoms

or

H;

L^N is a single bond

or

1 to 5 ω-amino acid moieties connected by peptide bonds, wherein the amino acid moieties contain 3 to 10 carbon atoms;

A^N is

a single bond

or

Xaa^1 Xaa^1 Pro

B is

Xaa^2 Xaa^1 Xaa^1 Xaa^2 Xaa^2 Xaa^1 Xaa^2

A^C is

a single bond

or

Pro Xaa^1 Xaa^1;

L^C is a single bond

or

1 to 5 ω-amino acid moieties connected by peptide bonds, wherein the amino acid moieties contain 3 to 10 carbon atoms;

and

Y is

a sulphur containing residue like Cys, the carboxyl group of which optionally can be substituted by -OAlk, -NH_2, -NHAlk or -$NAlk_2$, or -HN-$(CH_2)_n$-SH, with n = 2-11

or

an diamino acid like Lys, Orn, Dpr, or Dbu, optionally the side chain amino group of these diamino acids can be acylated by an acyl moiety with 2 to 22 C-atoms;

or

a dipeptide containing two diamino acids like Lys, Orn, Dpr, or Dbu, optionally one or both side chain amino groups of these diamino acids can be acylated by an acyl moiety with 2 to 22 C-atoms

or

OH;

and

Alk and Alk´

is independent of each other

a straight chain or branched alkyl with 1 to 11 C-atoms,

Pro can also be 3Hyp, or 4Hyp;

Xaa^1 is a hydroxy amino carbonic acid with 3 or 4 C-atoms, e.g. Ser, Thr, allo-Threonine, homo-Serine;

Xaa^2 is a 2-alkylglycine with C_1 - C_5 alkyl (straight chain or branched), e.g. Ala, Abu, Val, Ile, or Leu;

wherein either X or Y contain at least one sulphur atom,

and wherein only of residue A^N or A^C may represent a single bond.

2. Process for the preparation of a compound of formula I according to claim 1, wherein the building blocks are reacted in a procedure known in the art to form the given sequence.

3. Synthetic peptide layer consisting of one or several peptides of formula I in claim 1, which are covalently bonded via sulphur bridges of one of the terminal groups to a noble metal surface.

4. Synthetic cell membranes characterized in that they consist of a noble metal support which is covalently bonded to a peptide of formula I in claim 1, which via a sulphur bridge formed by one of the terminal groups of said peptide, characterized in that the other terminal group of said peptide is linked to lipid residues which in turn form, with liposomes where present, a membrane-analogous lipid bilayer.

5. Complex comprising at least one membrane protein which is inserted into a synthetic cell membrane according to claim 4.

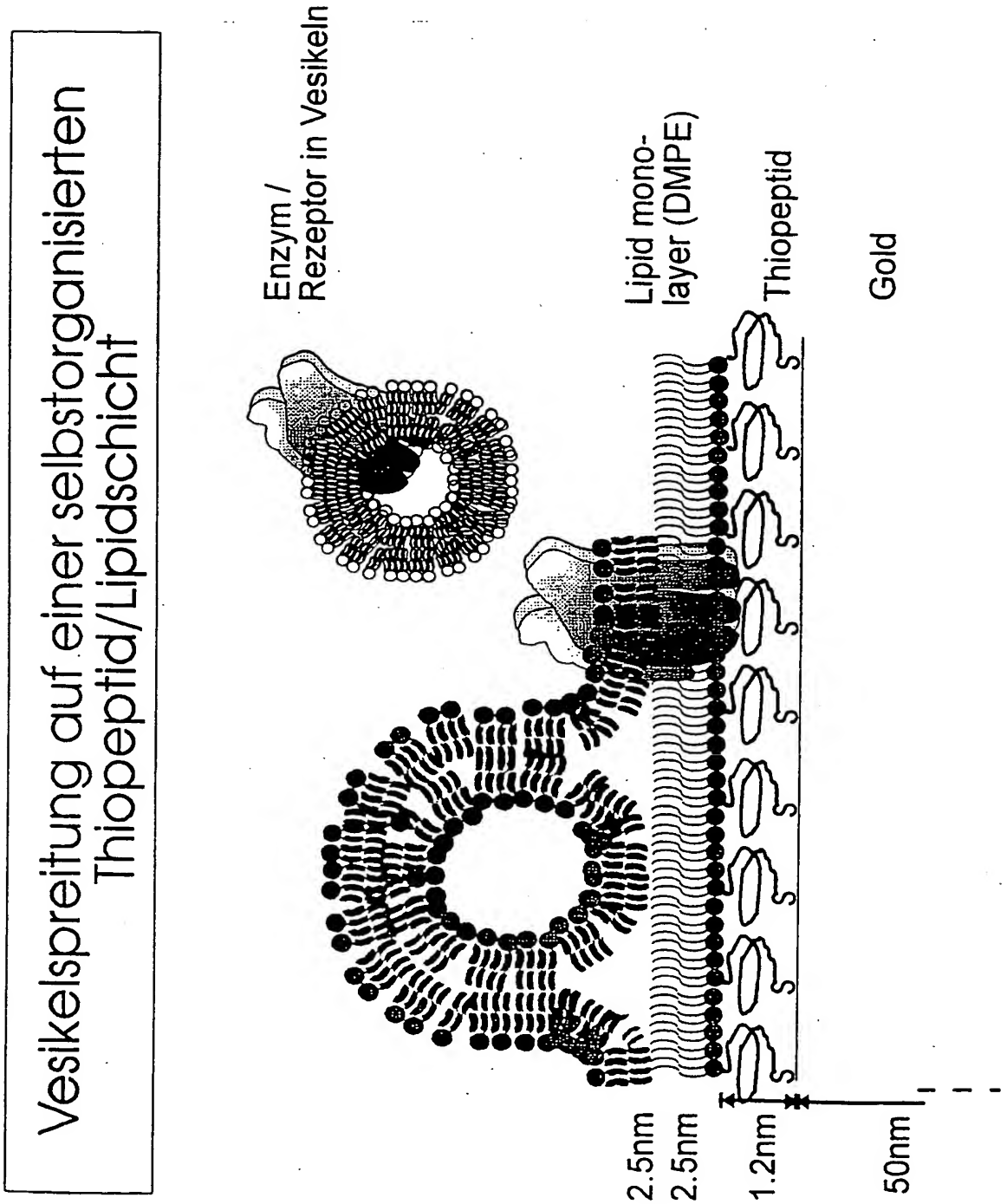
6. Process for the production of a complex according to claim 5 with the following steps:
   a) introducing a substrate coated with a noble metal into a solution of a peptide or a peptide analogous compound of formula I according to claim 1, containing a lipid component;
   b) forming a lipid bilayer, e.g. by adding liposomes;
   c) incorporating a membrane protein into the lipid bilayer of the previous step.

7. Biosensing device comprising electrical and/or optical measuring means and a complex of claim 5.

8. Use of a biosensing device according to claim 7 for receptor binding assays.

9. Use of a biosensing device according to claim 7 for investigating the activity of pharmaceuticals and crop protection agents.

Fig. 1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1.2nm
50nm

Fig. 2

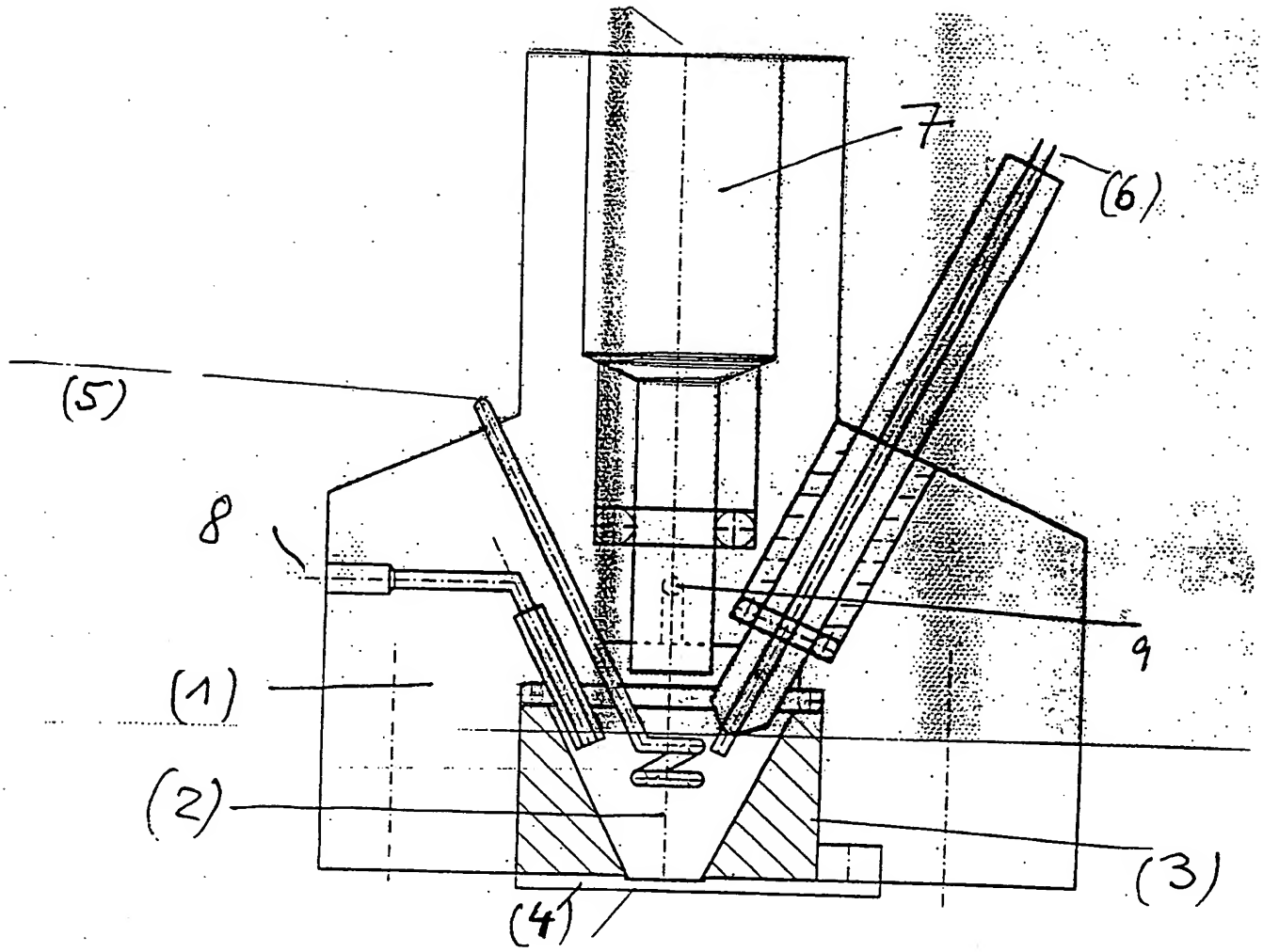

Fig. 3

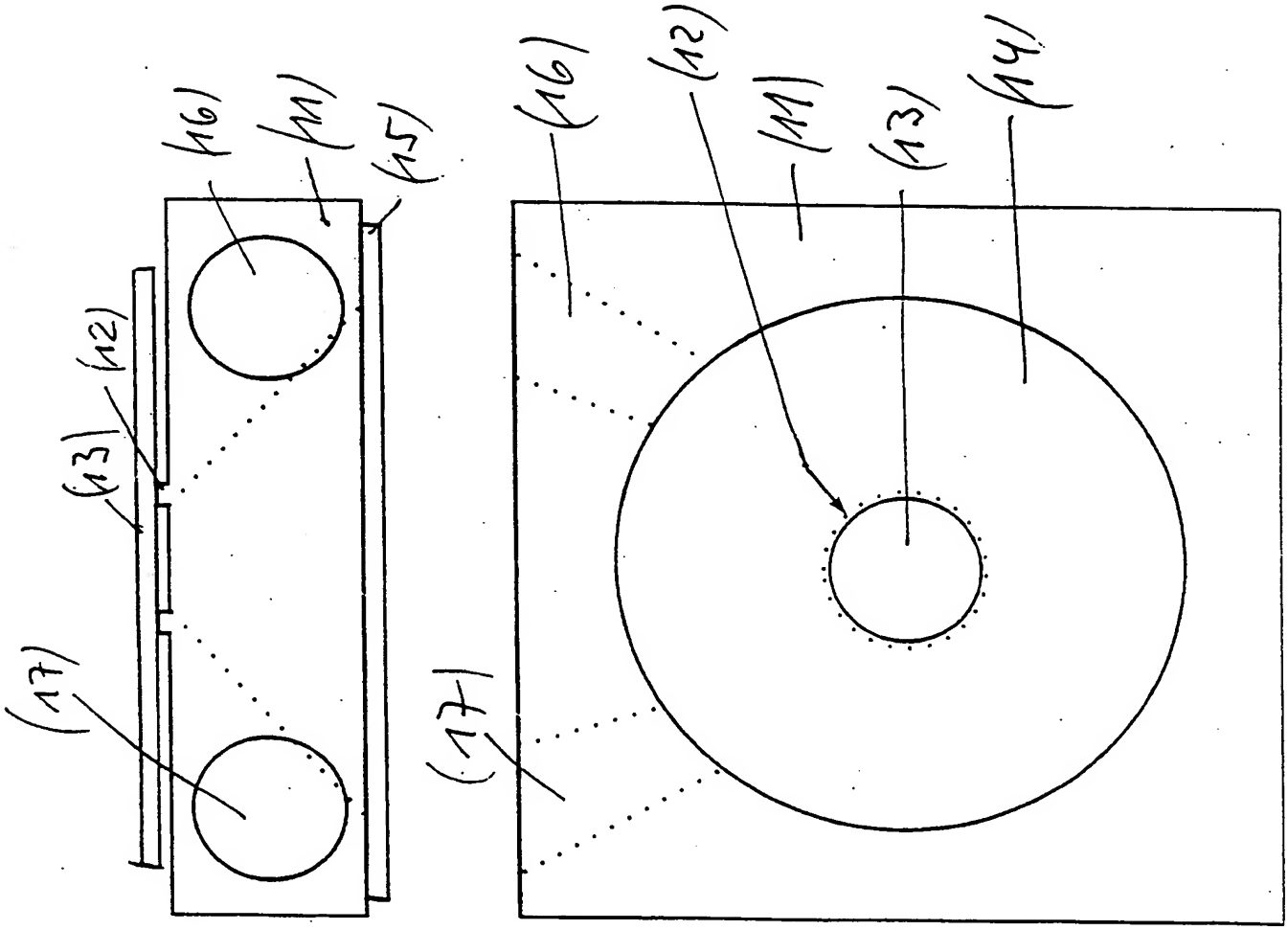

Fig. 4

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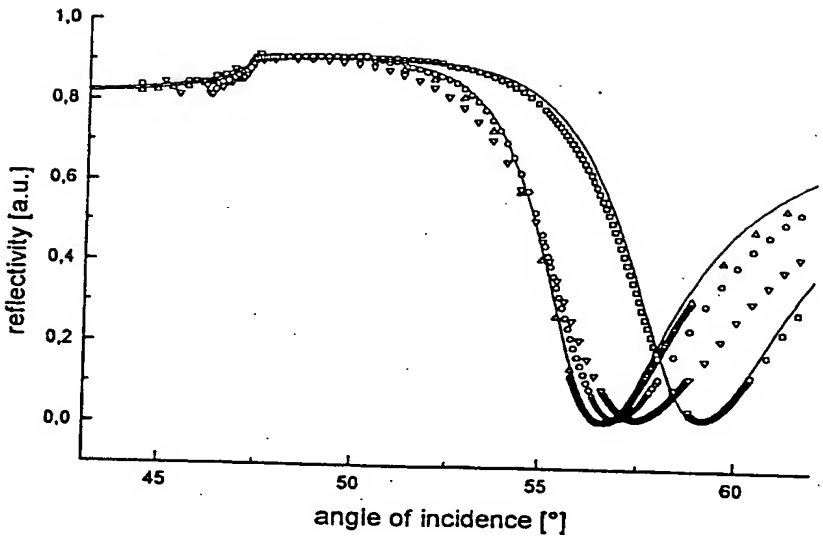
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b)

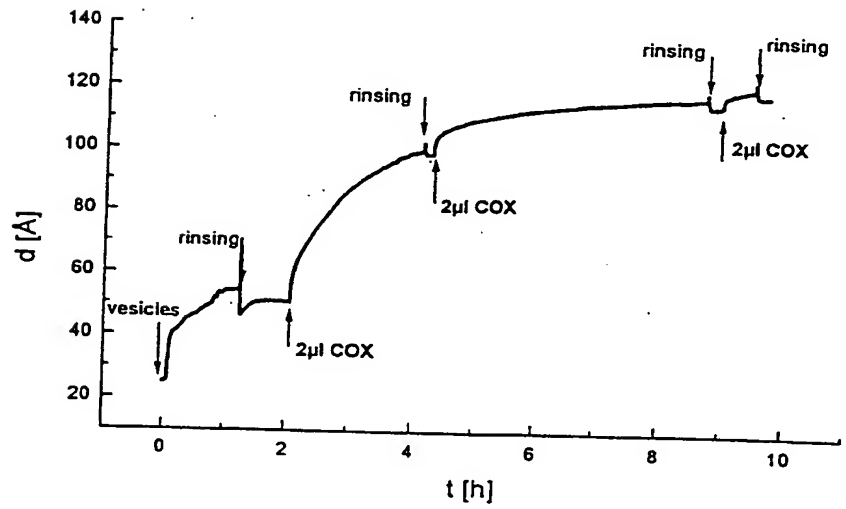
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Fig. 5

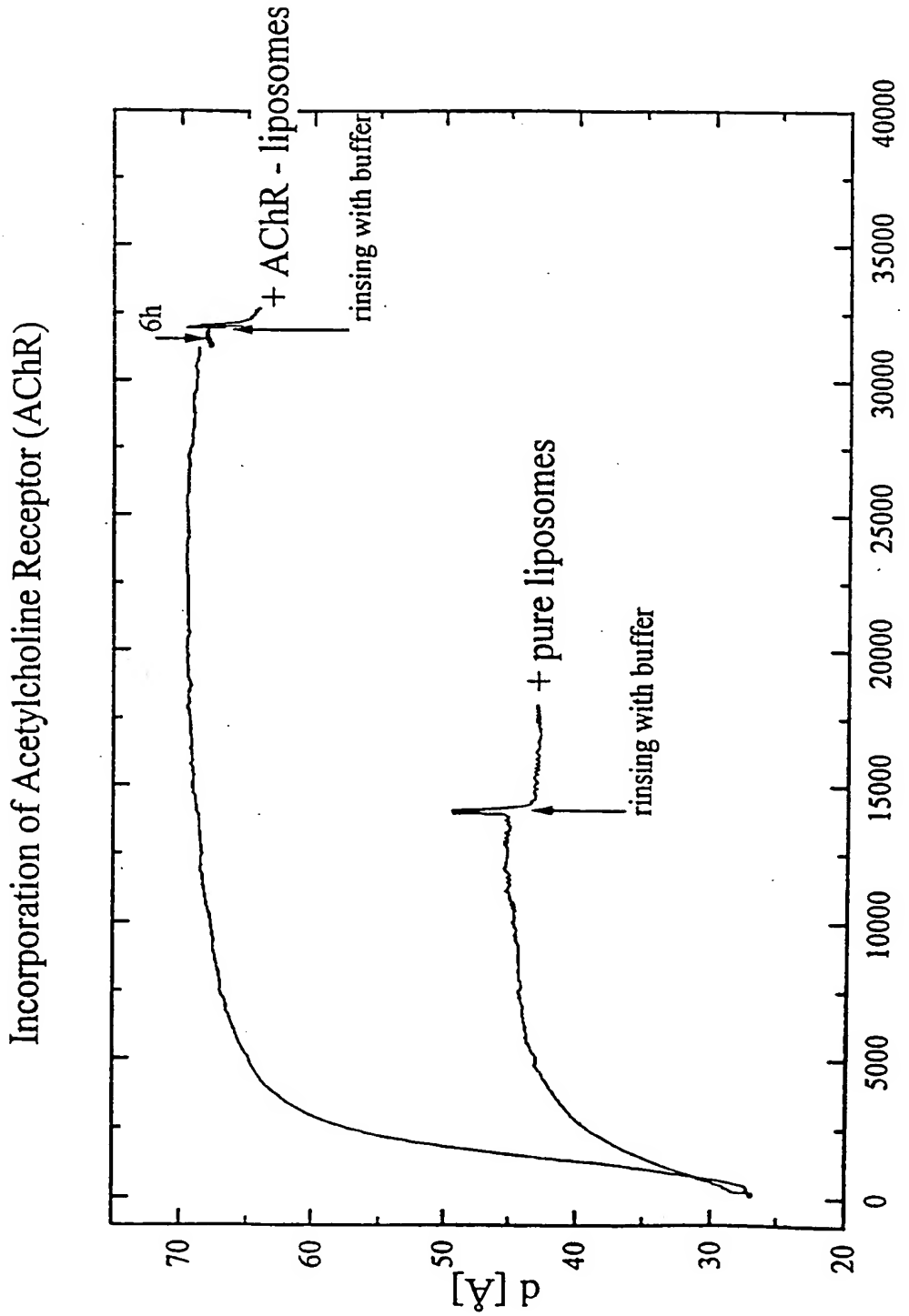
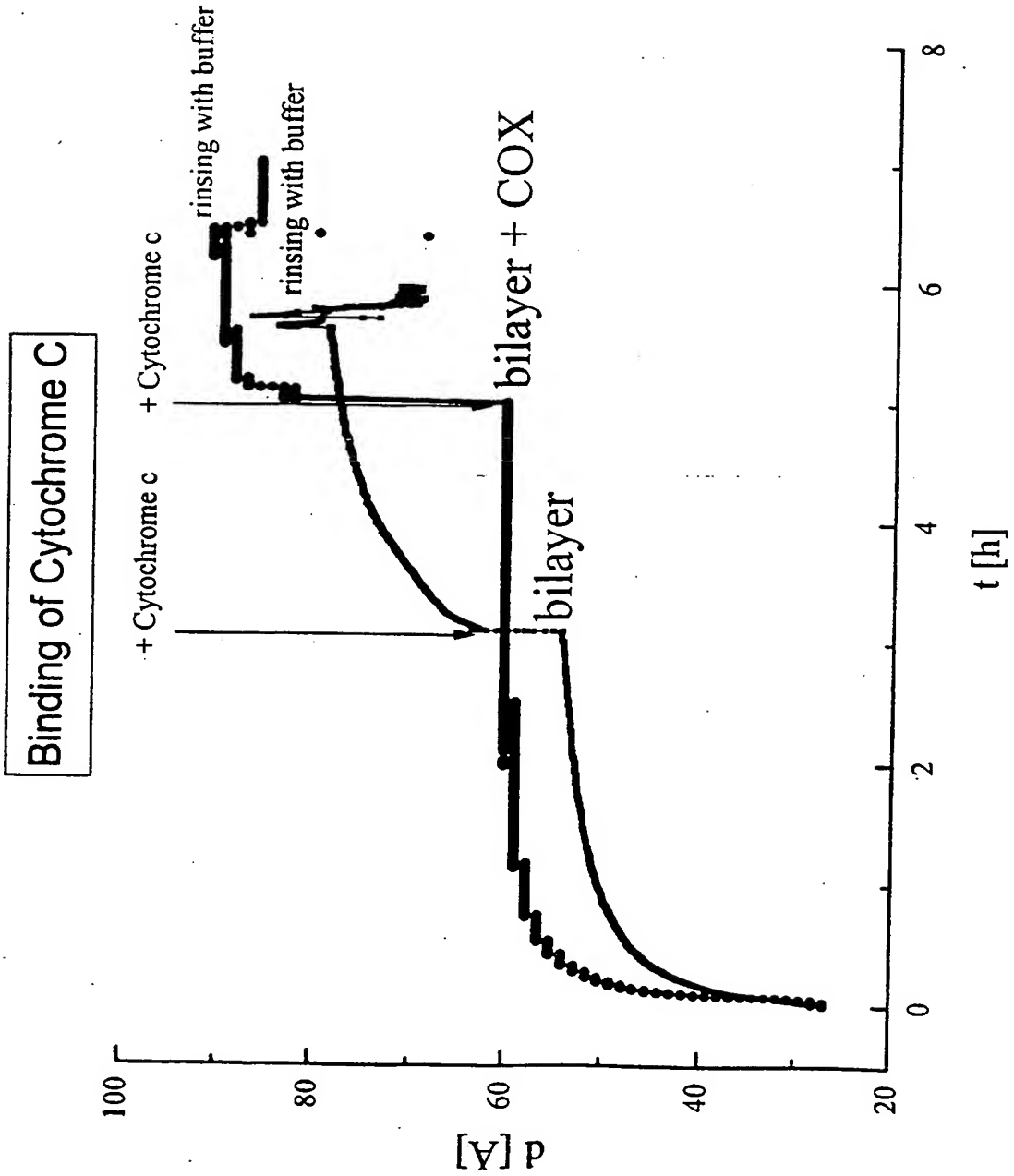


Fig. 6


Binding of Cytochrome C
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+ Cytochrome c
rinsing with buffer
rinsing with buffer
bilayer + COX
bilayer
d [Å]
t [h]
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80
60
40
20
0
2
4
6
8

Fig. 7

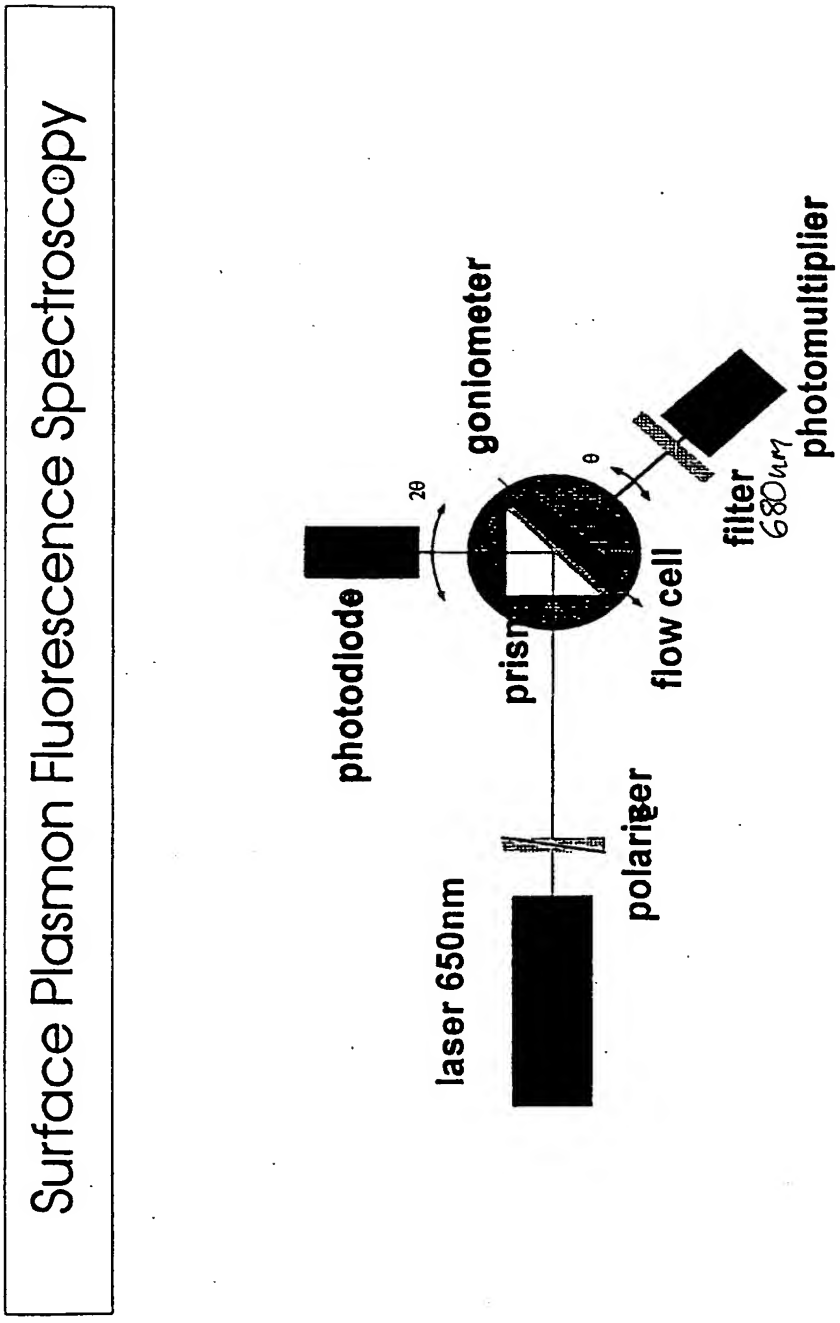


Fig. 8

Cytochrome C Oxidase - Reconstitution in a Peptide Supported Bilayer- Detection with Surface Plasmon Fluorescence Spectroscopy

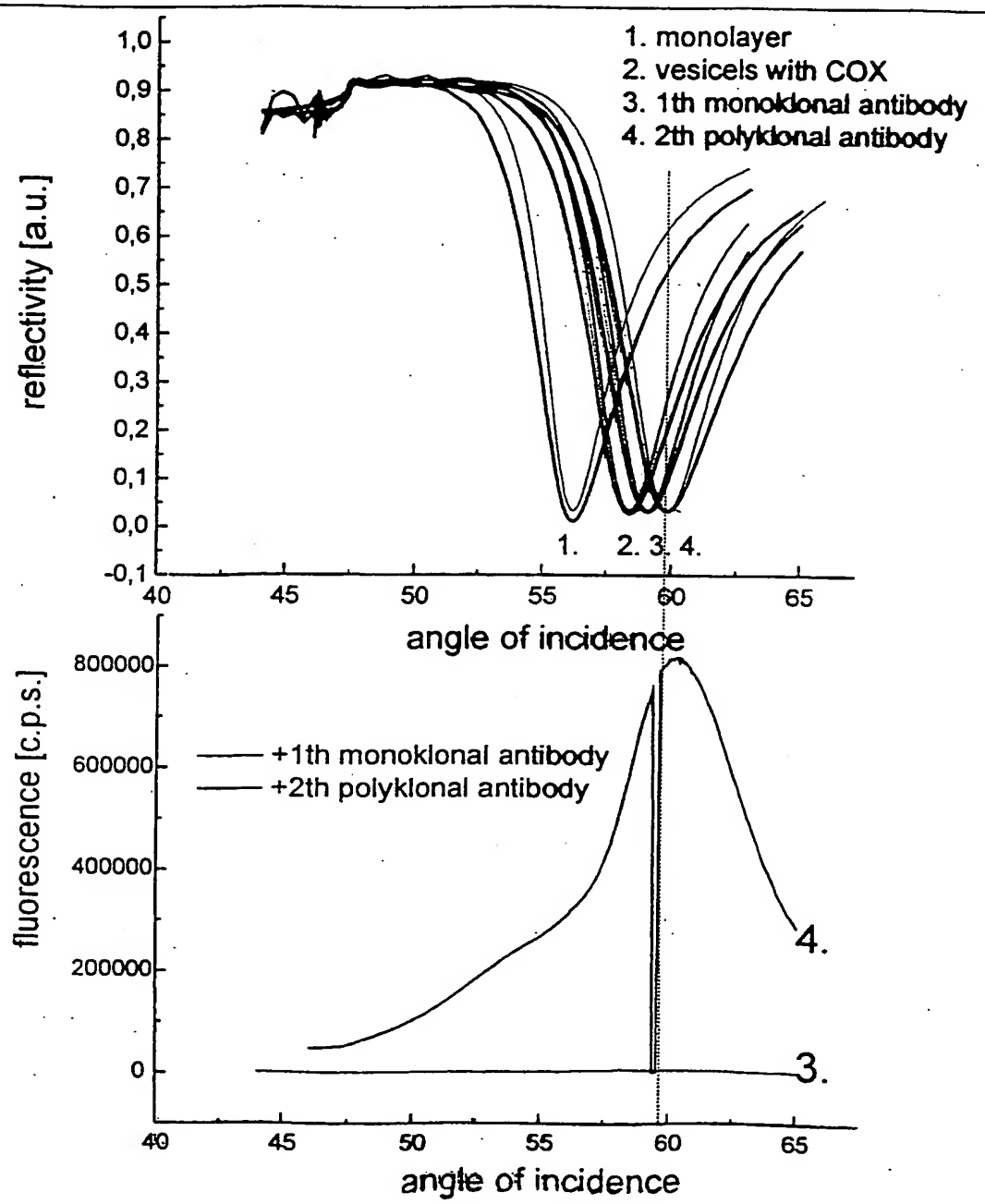


Fig. 9

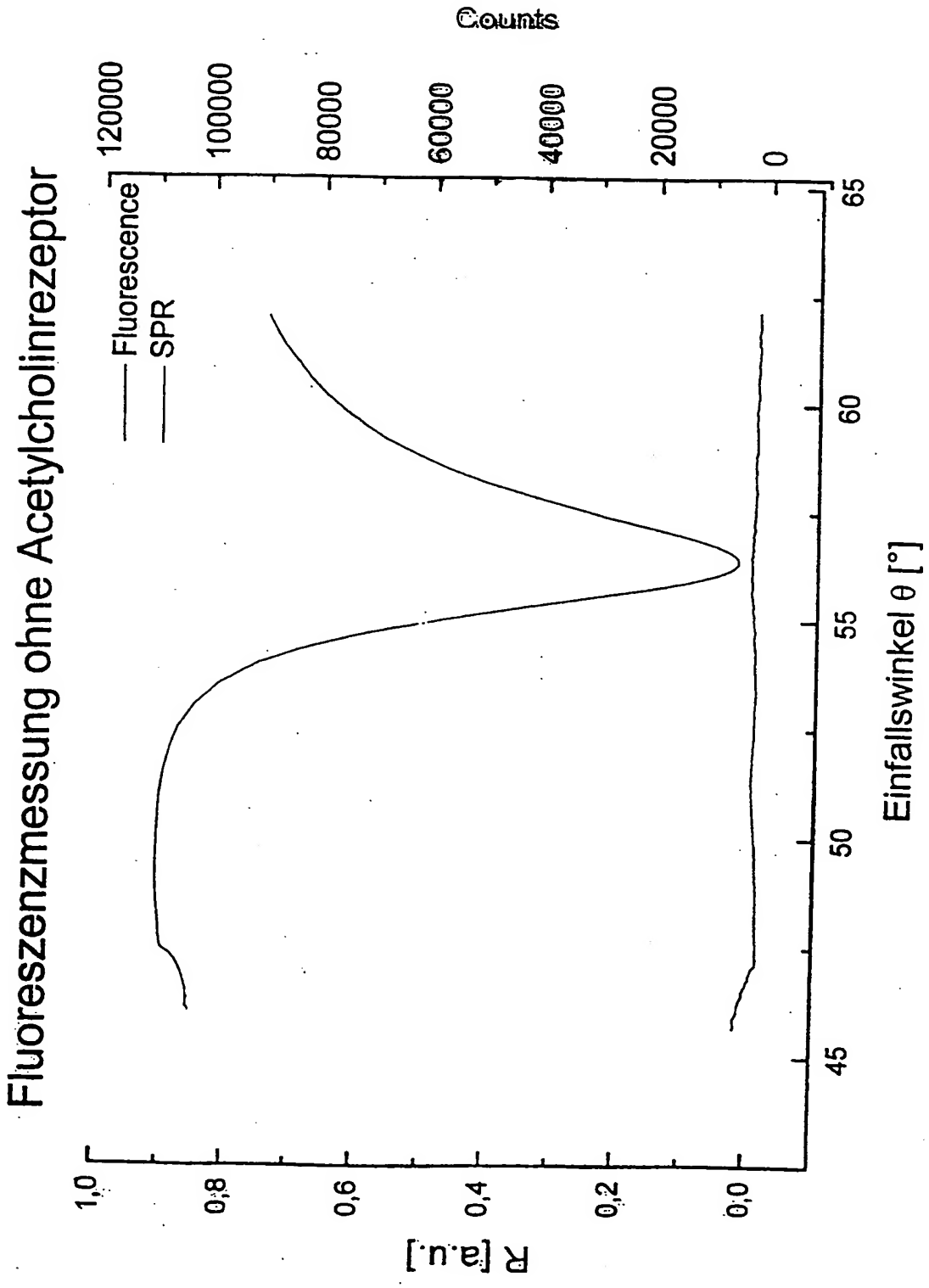

Fluoreszenzmessung ohne Acetylcholinrezeptor
Counts
120000
100000
80000
60000
40000
20000
0
Fluorescence
SPR
65
60
55
50
45
Einfallswinkel θ [°]
R [a.u.]
1,0
0,8
0,6
0,4
0,2
0,0

Fig. 10

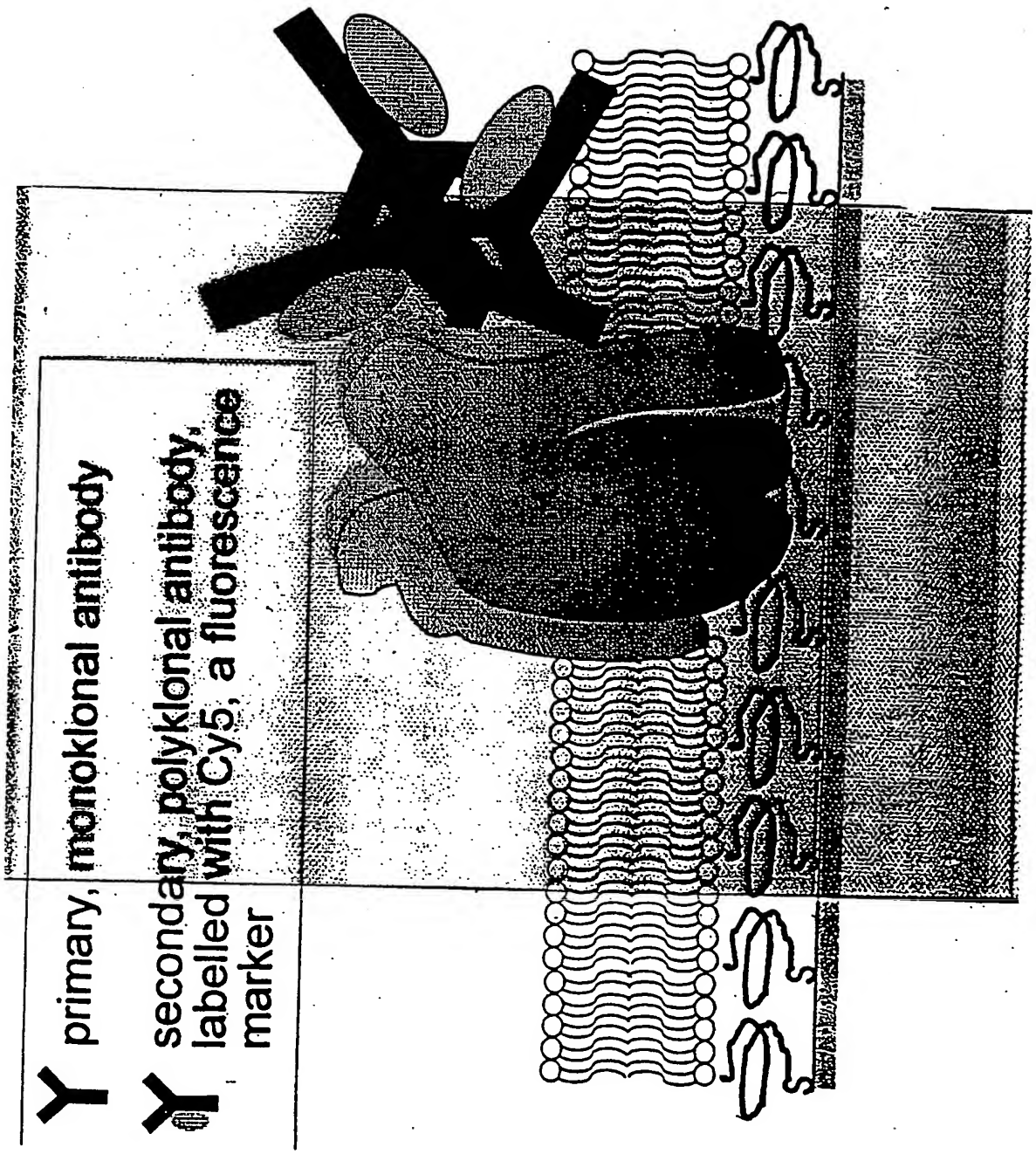

primary, monoklonal antibody
secondary, polyklonal antibody, labelled with Cy5, a fluorescence marker

Fig. 11

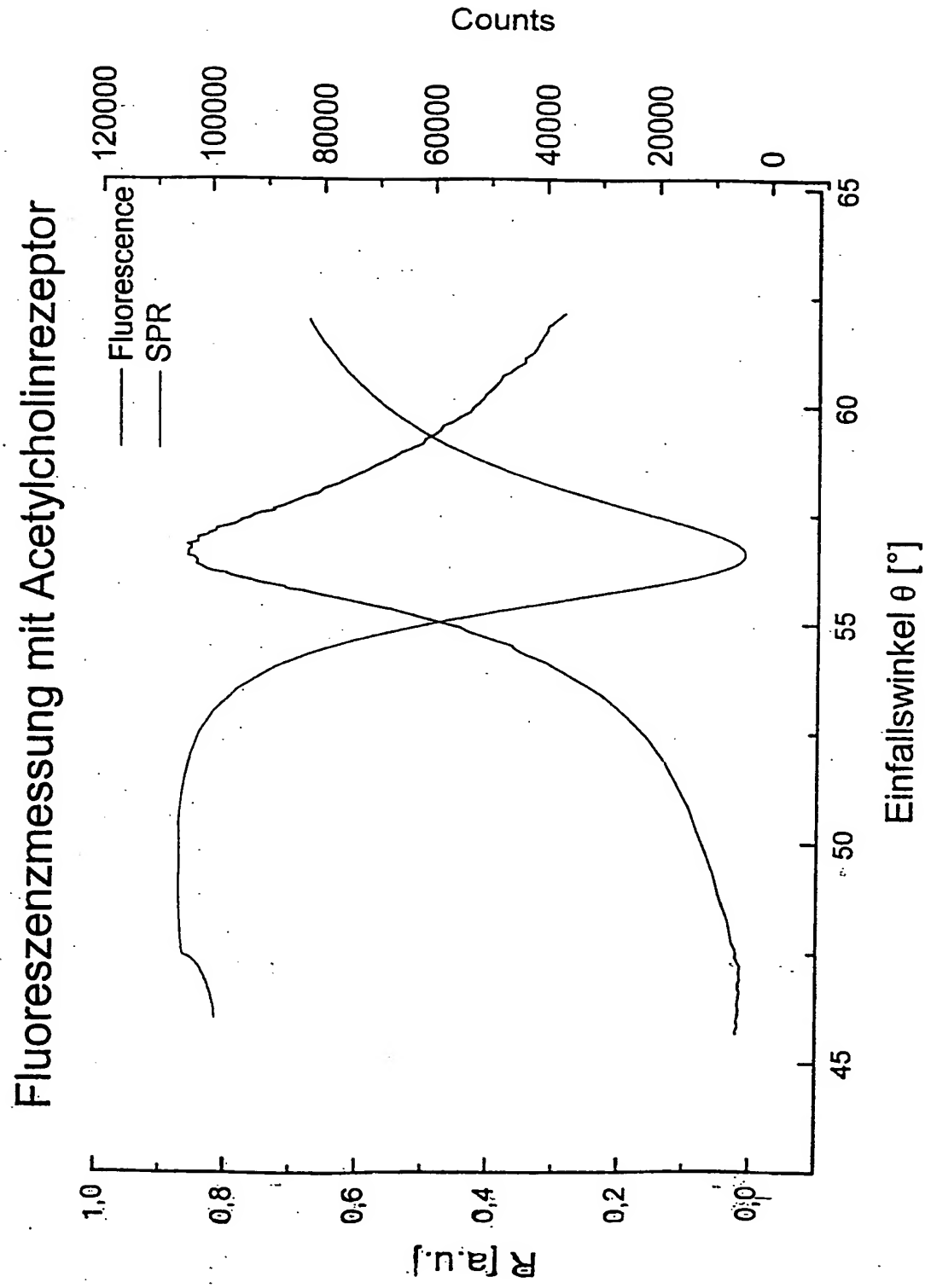
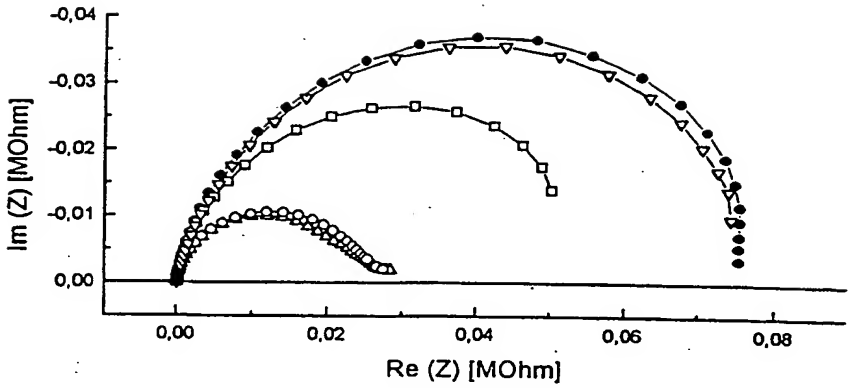

Fluoreszenzmessung mit Acetylcholinrezeptor
Counts
120000
100000
80000
60000
40000
20000
0
Fluorescence
SPR
R [a.u.]
1,0
0,8
0,6
0,4
0,2
0,0
45
50
55
60
65
Einfallswinkel θ [°]

Fig. 12

a)


-0,04
-0,03
-0,02
-0,01
0,00
Im (Z) [MOhm]
0,00
0,02
0,04
0,06
0,08
Re (Z) [MOhm]

b)

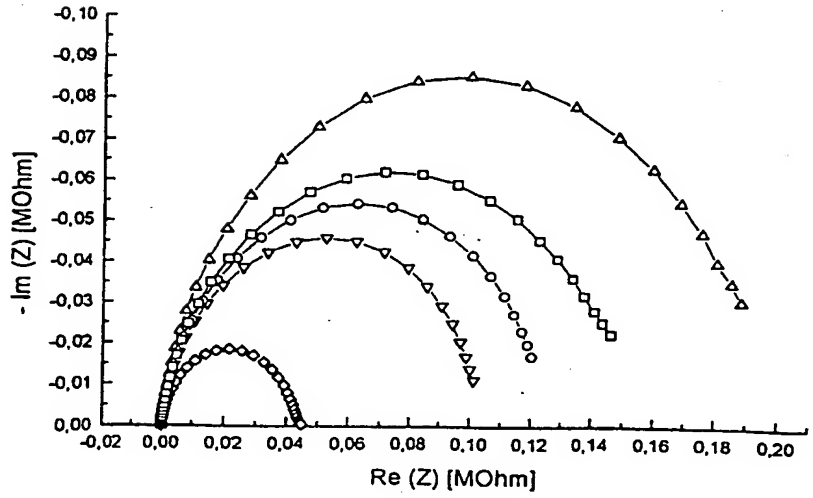

-0,10
-0,09
-0,08
-0,07
-0,06
-0,05
-0,04
-0,03
-0,02
-0,01
0,00
- Im (Z) [MOhm]
-0,02
0,00
0,02
0,04
0,06
0,08
0,10
0,12
0,14
0,16
0,18
0,20
Re (Z) [MOhm]

Fig. 13

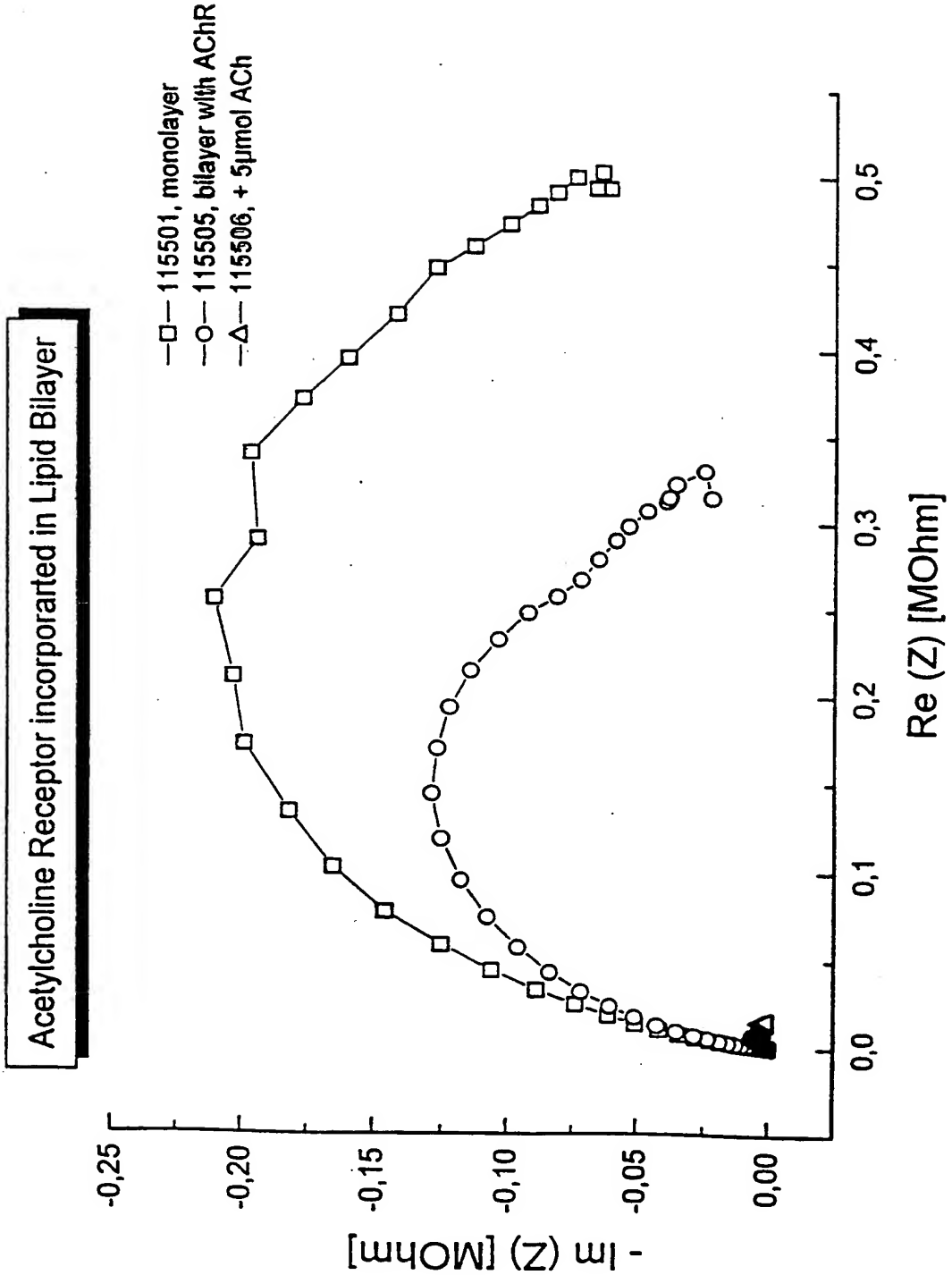
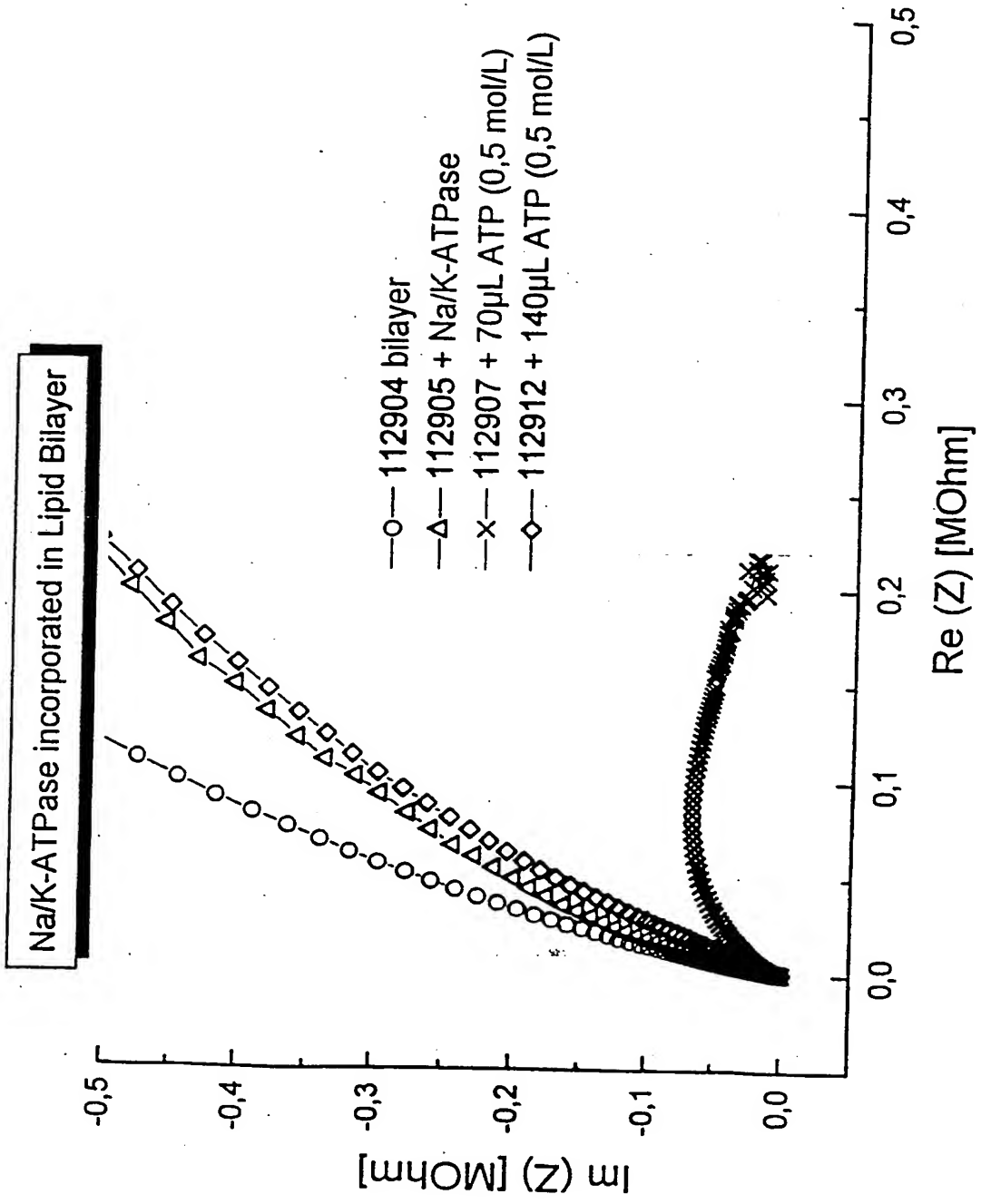


Fig. 14


Na/K-ATPase incorporated in Lipid Bilayer
112904 bilayer
112905 + Na/K-ATPase
112907 + 70µL ATP (0,5 mol/L)
112912 + 140µL ATP (0,5 mol/L)
Im (Z) [MOhm]
Re (Z) [MOhm]
-0,5
-0,4
-0,3
-0,2
-0,1
0,0
0,1
0,2
0,3
0,4
0,5

INTERNATIONAL SEARCH REPORT

Interi onal Application No
PCT/EP 98/06344

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K7/06 C07K7/02 G01N33/553 G01N33/68 C07K17/14

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	N. BUNJES ET AL.: "Thiopeptide-supported lipid layers on solid surfaces" LANGMUIR, vol. 13, November 1997, pages 6188-6194, XP002097603 see the whole document ---	1-9
X	WO 96 18645 A (MERCK PATENT GMBH ;NAUMANN RENATE (DE); JONCZYK ALFRED (DE)) 20 June 1996 see page 8; claims ---	1-9
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
23 March 1999	09/04/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cervigni, S

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INTERNATIONAL SEARCH REPORT

Inter ɔnal Application No
PCT/EP 98/06344

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	R. NAUMANN ET AL.: "Incorporation of membrane proteins in solid-supported lipid layers" ANGEWANDTE CHEMIE. INTERNATIONAL EDITION., vol. 34, no. 18, 1995, pages 2056-2058, XP002097604 WEINHEIM DE see figure 1 ---	
A	D. J VD HEUVEL ET AL: "Synthetic peptides as receptors in affinity sensors: a feasibility study" ANALYTICAL BIOCHEMISTRY, vol. 215, 1993, pages 223-230, XP002097192 see abstract; figures 1,3 ---	
A	T. KELLER ET AL: "Reversible oriented immobilization of histidine-tagged proteins on gold surfaces using a chelator thioalkane" SUPRAMOLECULAR SCIENCE, vol. 2, no. 3/04, 1995, pages 155-160, XP002082734 see abstract; figures 1,2 ---	
A	EP 0 441 120 A (YEDA RES & DEV) 14 August 1991 ---	
A	WO 90 05305 A (PHARMACIA AB) 17 May 1990 -----	

1

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter onal Application No
PCT/EP 98/06344

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		JP 4501607 T	19-03-1992
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